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Human Naive CD8 T Cells Down-Regulate Expression of the WNT Pathway Transcription Factors Lymphoid Enhancer Binding Factor 1 and Transcription Factor 7 (T Cell Factor-1) following Antigen Encounter In Vitro and In Vivo[1]

Tim Willinger,[2]* Tom Freeman,[†] Mark Herbert,[‡] Hitoshi Hasegawa,[§] Andrew J. McMichael,* and Margaret F. C. Callan[¶]

The transcription factors lymphoid enhancer binding factor 1 (LEF1) and transcription factor 7 (TCF7) (T cell factor-1 (TCF-1)) are downstream effectors of the WNT signaling pathway, which is a critical regulator of T cell development in the thymus. In this study, we show that LEF1 and TCF7 (TCF-1) are not only expressed in thymocytes, but also in mature T cells. Our data demonstrate that Ag encounter in vivo and engagement of the TCR or IL-15 receptor in vitro leads to the down-regulation of LEF1 and TCF7 (TCF-1) expression in human naive CD8 T cells. We further show that resting T cells preferentially express inhibitory LEF1 and TCF7 (TCF-1) isoforms and that T cell activation changes the isoform balance in favor of stimulatory TCF7 (TCF-1) isoforms. Altogether, our study suggests that proteins involved in the WNT signaling pathway not only regulate T cell development, but also peripheral T cell differentiation. *The Journal of Immunology,* **2006, 176: 1439–1446.**

The differentiation of T lymphocytes is mainly controlled by signals through their Ag-specific TCR. In the thymus, TCR signals regulate the development of a mature T cell repertoire via positive and negative selection. In the periphery, naive T cells (T_N)[3] further differentiate into effector and memory T cells when recognizing foreign Ags derived from infectious pathogens via their TCR (1). Apart from TCR signals and cytokines, other factors contributing to peripheral T cell differentiation are not well characterized at present.

In addition to TCR signals, major developmental pathways such as the WNT, Notch, and Hedgehog signaling pathways influence T cell development (2–4). Interestingly, there is recent evidence that these major developmental pathways also control the differentiation of peripheral T cells. Thus, Notch regulates the decision of CD4 T cells between the Th1 vs the Th2 fate (5), while the Hedgehog pathway can influence the proliferation and cytokine production of human peripheral CD4 T cells (6, 7). In contrast, it is currently unknown whether the WNT pathway has any role in mature T cells.

The canonical WNT signaling pathway is a critical regulator of stem cell function, e.g., it controls the maintenance and self-renewal of hemopoietic stem cells (8–11). Furthermore, dysregulation of the WNT pathway commonly occurs in human cancers (11). In the absence of a WNT signal, cytoplasmic β-catenin is phosphorylated and targeted for degradation by the proteasome (3, 11). WNT signaling allows β-catenin to escape proteasomal degradation and to translocate to the nucleus. In the nucleus, β-catenin interacts with members of the lymphoid enhancer binding factor (LEF)/T cell factor (TCF) family of transcription factors (LEF1, TCF7 (TCF-1), TCF7L1 (TCF-3), and TCF7L2 (TCF-4)) to activate the transcription of WNT target genes such as c-*myc* and *cyclin D1* (11). In the absence of WNT signaling, i.e., when not interacting with β-catenin, LEF/TCF family members act as transcriptional repressors by recruiting Groucho repressor proteins (12).

LEF1 and TCF7 (TCF-1) share common protein motifs, in particular, the C-terminal HMG domain of both proteins is responsible for DNA binding, while a β-catenin-binding domain at the N terminus mediates the interaction with β-catenin (12). Interestingly, there are multiple LEF1 and TCF7 (TCF-1) protein isoforms with distinct functional properties (13–17). In addition to the stimulatory full-length isoforms, there are N-terminally truncated isoforms that are without the β-catenin-binding domain (referred to as ΔCTNNB) but retain the ability to interact with Groucho repressors (12). Importantly, these truncated isoforms can function in a dominant-negative manner in the WNT signaling pathway as has been demonstrated for ΔCTNNB isoforms of LEF1 (17, 18), TCF7 (TCF-1) (19, 20), and the *Xenopus* homolog of LEF1/TCF7 (TCF-1) (21). Finally, there are also LEF1 and TCF7 (TCF-1) isoforms with alternative C-termini (termed tails) known as N- or B-tailed isoforms. Currently, very little is known about the different functional properties of these multiple LEF1 and TCF7 (TCF-1) isoforms in T cells.

*Medical Research Council Human Immunology Unit, Weatherall Institute of Molecular Medicine, Oxford, United Kingdom; [†]Medical Research Council Rosalind Franklin Centre for Genomics Research, Wellcome Trust Genome Campus, Hinxton, Cambridge, United Kingdom; [‡]The Neonatal Unit, Women's Centre, John Radcliffe Hospital, Oxford, United Kingdom; [§]University School of Medicine, Shigenobu, Ehime, Japan; and [¶]Department of Rheumatology, Division of Medicine, Imperial College London, Chelsea and Westminster Hospital, London, United Kingdom

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[2] Address correspondence and reprint requests to Dr. Tim Willinger, Medical Research Council Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe, Oxford, U.K. E-mail address: TimW@hammer.imm.ox.ac.uk

[3] Abbreviations used in this paper: T_N, naive T cell; CB, cord blood; LEF1, lymphoid enhancer binding factor 1; qRT-PCR, quantitative RT-PCR; TCF7 (TCF-1), transcription factor 7 (T cell factor 1); T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{EMRA}, effector memory RA T cell; CD62L, CD62 ligand.

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Knockout and transgenic studies in mice have clearly shown a redundant and β-catenin-dependent role of LEF1 and TCF7 (TCF-1) in T cell development (3). TCF7 (TCF-1)$^{-/-}$ mice have impaired T cell development with a partial block at the intermediate single-positive to double-positive transition due to reduced thymocyte proliferation and survival (22–24). Although T cell development is normal in LEF1 $^{-/-}$ mice, B cell development is impaired (25). Thymocytes from TCF7 (TCF-1)$^{-/-}$ LEF1 $^{-/-}$ mice show a profound block at the intermediate single-positive stage with neither double-positive nor single-positive thymocytes present, and consequently no mature T cells in the periphery (26). Taken together, the WNT-β-catenin-LEF1/TCF7 (TCF-1) axis plays a pivotal role in T cell development. However, it is unknown whether LEF1, TCF7 (TCF-1), and the WNT pathway have a specific function in peripheral T cells.

Therefore, we undertook a detailed analysis of the expression of LEF1 and TCF7 (TCF-1) in human peripheral T cells. We found that both LEF1 and TCF7 (TCF-1) are expressed in mature CD8$^+$ T_N and that their expression is down-regulated following TCR or IL-15R engagement in vitro and Ag encounter in vivo. Furthermore, T cell activation changed the balance of stimulatory vs inhibitory LEF1 and TCF7 (TCF-1) isoforms. Our results suggest that the WNT pathway, in addition to its well-known role in T cell development, is likely to be involved in regulating peripheral T cell differentiation.

Materials and Methods

Isolation of CD8 T cell subsets

For microarray and quantitative RT-PCR (qRT-PCR) experiments, CD8 T cell subsets were isolated from healthy donors in accordance with institutional ethics approval as previously described (27). CD8 T cells were sorted into either T_N (CCR7$^+$CD45RA$^+$) and effector memory T cells/effector memory RA T cells ($T_{EM/EMRA}$; CCR7$^-$CD45RA$^{+/-}$) (microarray data set 1), or T_N (CCR7$^+$CD45RA$^+$), central memory T cells (T_{CM}; CCR7$^+$CD45RA$^-$), T_{EM} (CCR7$^-$CD45RA$^-$), and T_{EMRA} (CCR7$^-$CD45RA$^+$) populations (microarray data set 2).

Microarray gene expression analysis

RNA extraction and labeling was performed as previously described (27, 28). For microarray data set 1, total RNA was pooled from several donors (replicate pool 1: $n = 10$; replicate pool 2: $n = 6$) and hybridized to Affymetrix HG-U95Av2 arrays. Two independent microarray experiments were performed with RNA from CD8$^+$ T_N and $T_{EM/EMRA}$ for data set 1. For microarray data set 2 (described in Ref. 27), total RNA from individual donors was used and hybridized to Affymetrix HG-U133 plus 2.0 arrays (Affymetrix). Four independent microarray experiments were performed with RNA from CD8$^+$ T_N, T_{CM}, T_{EM}, and T_{EMRA} for data set 2. We used GCOS software (Affymetrix) and the software package BRB-ArrayTools for data analysis and the identification of differentially expressed genes between CD8 T cells subsets (27).

qRT-PCR analysis

qRT-PCR was conducted on cDNA from the indicated CD8 T cell populations with the 5′ nuclease/TaqMan assay. Briefly, we prepared cDNA from ~1 μg of DNase-treated total RNA using the SuperScript First-Strand Synthesis System (Invitrogen Life Technologies). We then performed quantitative PCR in a final volume of 25 μl with 300 nM of the forward and reverse primers and 100–250 nM of the fluorogenic TaqMan probes (Eurogentec) using 2× quantitative PCR Mastermix Plus (Eurogentec). Reactions were run on an ABI Prism 7700 Sequence Detection System machine (Applied Biosciences) in triplicate (initial steps: 50°C/2 min and 95°C/10 min, followed by 40 cycles: 95°C/15 s and 60°C/1 min) The following primers and probes were used: 1) LEF1: forward, TGA CAGCTGCCTACATCTGAAAC; reverse, GCTGCCTTGGCTTTGCAC; probe: FAM-TGGTGGAAAACGAAGCTCATTCCCAA-TAMRA. LEF1 primers and probes target exon 11/exon 12 and are specific for all LEF1 N-tail isoforms. 2) TCF7 (TCF-1): forward, TGCAGCTATACCCAG GCTGG; reverse, CCTCGACCGCCTCTTCTTC; probe: FAM-TCCCG TAGTTGTCCCGCGCTG-TAMRA. TCF7 (TCF-1) primers and probes target exon 7/exon 8 and are specific for all TCF7 (TCF-1) isoforms. 3) hypoxanthine phosphoribosyltransferase: forward, GACTTTGCTTTCCT TGGTCAGG; reverse, AGTCTGGCTTATATCCAACACTTCG; probe: FAM-TTTCACCAGCAAGCTTGCGACCTTGAC-TAMRA. All probes span exon-intron junctions. We applied the comparative threshold cycle method for relative quantification of mRNA expression according to the manufacturer's recommendations. Validation experiments demonstrated that the amplification efficiencies of LEF1 and TCF7 (TCF-1) were equal to that of the endogenous control hypoxanthine phosphoribosyltransferase.

Intracellular FACS staining

We analyzed intracellular expression of LEF1 and TCF7 (TCF-1) in CD8 T cell subsets using the methanol permeabilization protocol as previously described (27). LEF1 and TCF7 (TCF-1) were detected by indirect staining using pretitrated mAbs REMB6 (Oncogene Research Products) and 7H3 (Upstate Biotechnology), respectively. Briefly, 2 μg of primary mAb was added to 2×10^6 cells and incubated for 1 h at room temperature. After washing, this was followed by staining with PE-conjugated rabbit anti-mouse Ab (DakoCytomation) for 1 h at room temperature. Cells were subsequently washed in blocking buffer (PBS containing 2% mouse serum) before surface staining with directly conjugated mAbs specific for CD62 ligand (CD62L), CD45RA, and CD8 (all BD Biosciences). CD62L was used instead of CCR7 as a surface marker because CCR7 staining was compromised following methanol permeabilization (27).

Stimulation of cord blood (CB) CD8 T cells in vitro

We obtained CB samples from the John Radcliffe Hospital maternity unit, upon written consent and approval by the local Medical Ethics Committee. CB CD8 T cells were isolated by immunomagnetic selection as described above. Phenotyping was conducted using mAbs specific for CCR7 (R&D Systems), CD45RA (BD Biosciences), CD8 (BD Biosciences), CD3, CD25, and HLA class II (all DakoCytomation). We stimulated CB CD8 T cells ($1–2 \times 10^6$/ml) with either plate-bound anti-CD3 mAb OKT3 (1 μg/well), IL-15 (50 ng/ml), or TGFβ1 (3 ng/ml) in 24-well plates for the indicated time points. All cytokines were from obtained R&D Systems. Cells were cultured in complete medium (RPMI 1640 supplemented with 10% FCS, 1% sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37°C in 5% CO_2. Alternatively, CD8 T cells (1×10^6) were cocultured with irradiated allogeneic EBV-transformed B cells at a 1:1 ratio. From day 3 onward, CD3$^+$ cells represented >95% of live cells in these cocultures, i.e., the stimulator B cells had practically disappeared.

Cloning of LEF1 mRNA isoforms from primary CD8 T cells

Total RNA was extracted from primary CD8 T cells using TRI Reagent (Sigma-Aldrich) and cDNA was prepared as described above for the qRT-PCR experiments. RT-PCR was performed with the following primers: forward primers, CAGCGGAGCTCAGATTACAGAG (full-length isoforms) and ACTCGAGCTCTTCCGGGTACATAATG (ΔCTNNB isoforms); reverse primers, CTTCGAATTCCACCATGTTTCAGATG (N-tail isoforms) and GTCAGAATTCCTTTGGCGTCGACTG (B-tail isoforms). PCR products were cloned into the vector pIRES2-EGFP (BD Clontech) followed by DNA sequencing.

Western blot analysis

We prepared protein lysates from ~3 to 5×10^6 CD8 T cells by washing the cells in PBS and resuspending them in an equal volume of 2× sample buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 8% 2-ME, 0.2% bromphenol blue, and 20% glycerol). After sonication for 2×20 s and boiling for 5–10 min, protein samples were separated by SDS-PAGE and gels blotted to nitrocellulose membranes. We performed immunodetection of LEF1 and TCF7 (TCF-1) with the REMB6 mAb (Exalpha) at a 1/500 dilution and with the 7H3 mAb (Upstate Biotechnology) at a 1/1000 dilution, respectively. This was followed by incubation with secondary HRP-conjugated anti-mouse Ig (DakoCytomation) and signal detection with ECL reagent (BD Amersham). Blots were stripped by incubating the membrane at 50°C for 30 min in stripping buffer (62.5 mM Tris-HCl (pH 6.7), 2% SDS, and 100 mM 2-MEl) and reprobed with anti-β-actin mAb AC-15 at a 1/5000 dilution (Sigma-Aldrich).

Statistical analysis

A two-sample, two-tailed t test assuming unequal variances was used to determine the significance of differences in mRNA expression between two groups ($\alpha = 0.05$). For multigroup comparisons, we applied one-way ANOVA with post hoc testing using Tukey's significant difference test ($\alpha = 0.05$).

Results

CD8$^+$ T_N down-regulate expression of LEF1 and TCF7 (TCF-1) upon Ag encounter in vivo

We used microarray technology to screen for genes that are differentially expressed between human T_N and Ag-primed CD8 T cells. Our first, exploratory, microarray data set compared gene expression in purified CD8$^+$ T_N (CCR7$^+$CD45RA$^+$) and CD8$^+$ $T_{EM/EMRA}$ (CCR7$^-$CD45RA$^{+/-}$) populations using RNA pooled from several donors. We also generated a more detailed second data set that analyzed the gene expression profiles of CD8$^+$ T_N (CCR7$^+$CD45RA$^+$) in relation to that of CD8$^+$ T_{CM} (CCR7$^+$CD45RA$^-$), T_{EM} (CCR7$^-$CD45RA$^-$), and T_{EMRA} (CCR7$^-$CD45RA$^+$) subsets. Technical advances allowed us to use RNA from individual donors instead of pooled RNA and to perform a greater number of replicate experiments (four instead of two) for the second data set. Furthermore, gene chips (HG-U133 Plus 2.0 instead of HG-U95Av2) with a greater number of probes and greater coverage of the human genome were available for this data set. Using this data set, we have previously investigated the molecular relationships between T_N and memory (Ag-primed) CD8 T cell subsets and the molecular basis for their different functional properties (27). We now aimed to examine selected differentially expressed genes in more detail to gain further insight into the molecular basis of CD8 T cell behavior.

We identified *LEF1* as a gene highly expressed in T_N, compared with Ag-primed CD8 T cell subsets. In both microarray data sets, *LEF1* was found to be in the top three most differentially expressed genes when comparing the different mature CD8 T cell populations (Table I). Indeed, within data set 2, probe identifications for *LEF1* accounted for two of the top three differentially expressed genes. LEF1 mRNA levels were 5- to 10-fold higher in T_N, compared with the three Ag-experienced subsets, with T_{CM} expressing LEF1 more strongly than T_{EM} and T_{EMRA} (Fig. 1*A*). Interestingly, we found that *TCF7* (*TCF-1*) was also expressed at higher levels in T_N, compared with T_{CM}, T_{EM}, and T_{EMRA} (Fig. 1*B*).

qRT-PCR experiments confirmed the differential expression of LEF1 and TCF7 (TCF-1) in T_N and Ag-primed CD8 T cell subsets (Fig. 1). We also examined LEF1 and TCF7 (TCF-1) protein expression in peripheral CD8 T cell subsets by intracellular flow cytometry. Importantly, as shown in Fig. 2, T_N (and T_{CM}) expressed significantly higher levels of both LEF1 and TCF7 (TCF-1) protein than T_{EM} and T_{EMRA} ($p < 0.05$, one-way ANOVA/Tukey's post hoc test). In summary, we found that the WNT pathway effectors LEF1 and TCF7 (TCF-1) are expressed in peripheral T cells and that LEF1 is the most differentially expressed transcription factor between T_N and Ag-primed CD8 T cell subsets.

Expression of LEF1 and TCF7 (TCF-1) in CD8$^+$ T_N is down-regulated by TCR signals and IL-15 in vitro

We studied the regulation of LEF1 mRNA expression in peripheral CD8 T cells in vitro by qRT-PCR. We observed down-regulation of LEF1 mRNA expression in CD8 T cells in response to TCR triggering (Fig. 3*A*). This down-regulation was rapid (within 12 h) and persisted for >48 h. Stimulation with homeostatic cytokines, such as IL-15, also lead to a persistent decrease in LEF1 mRNA levels (Fig. 3*B*), with IL-2 having a similar effect (data not shown). In contrast, stimulation with TGFβ1 increased LEF1 expression in CD8 T cells, compared with the medium control (Fig. 3*C*).

To investigate the expression of LEF1 and TCF7 (TCF-1) in CD8$^+$ T_N, we used CD8 T cells isolated from CB. CB CD8 T cells have a predominant CCR7$^+$CD45RA$^+$ phenotype similar to adult T_N (Fig. 4*A*) with some CCR7$^-$CD45RA$^+$ cells also present. Importantly, it has been shown that CB T cells (including the CCR7$^-$CD45RA$^+$ subset) are functionally naive (29). CB CD8 T cells showed strong up-regulation of activation markers such as CD25 (IL-2Rα; Fig. 4*A*) and HLA class II (data not shown) in response to TCR (allogeneic) stimulation. At later time points, poststimulation CD8 T cells converted back to a resting state as shown by the absence of activation marker expression (Fig. 4*A*). Similar to bulk peripheral CD8 T cells, we observed down-regulation of LEF1 mRNA expression in CD8$^+$ T_N (from CB) following TCR stimulation as measured by qRT-PCR (Fig. 4*B*). Importantly, at the time points examined (more than day 3) allogeneic stimulator B cells had practically disappeared from the T cell-B cell cocultures. Interestingly, after the initial down-regulation, there was a progressive increase in LEF1 mRNA expression at

Table I. *LEF1 is among the 10 most differentially expressed genes between T_N and Ag-primed CD8 T cell subsets*[a]

No.	GenBank Identification	Gene Symbol	Description	T_N	$T_{EM/EMRA}$		
Microarray data set 1							
1	AB018295	NY-REN-7	NY-REN-7 Ag	93.0	6.6		
2	M95178	ACTN1	Actinin α1	376.7	29.4		
3	AL049409	LEF1	Lymphoid enhancer-binding factor-1	853.2	65.3		
4	D90144	CCL3	MIP-1 α	66.4	491.0		
5	U11276	KLRB1	CD161	232.5	1155.1		
6	L31584	CCR7	CCR7	1385.7	238.6		
7	X76220	MAL	T lymphocyte maturation-associated protein	1242.9	106.9		
8	AJ223603	C4.4A	GPI-anchored metastasis-associated protein homolog	367.0	41.2		
9	AA478904	KLF7	Kruppel-like factor-7	189.2	38.0		
10	X02910	TNF	TNF-α	93.9	704.7		
No.	GenBank Identification	Gene Symbol	Description	T_N	T_{CM}	T_{EM}	T_{EMRA}
Microarray data set 2							
1	AA992805	LEF1	Lymphoid enhancer-binding factor-1	421.5	52.4	29.8	28.8
2	AI082078	ACTN1	Actinin, α1	928.9	47.4	25.5	41.4
3	AF294627	LEF1	Lymphoid enhancer-binding factor-1	1666.5	208.2	80.8	111.1
4	NM_014795	ZFHX1B	Zinc finger homeobox 1b	20.8	127.0	405.6	708.2
5	NM_006144	GZMA	Granzyme A	98.3	503.9	2240.4	2429.5
6	AI636759	SLC15A4	Solute carrier family 15, member 4	108.3	191.6	531.6	1020.8
7	AI356412	LYN	v-yes-1 Yamaguchi sarcoma viral-related oncogene homolog	22.0	23.6	85.0	313.1
8	BC004344	MGC29816	Hypothetical protein MGC29816	1181.2	240.1	170.6	169.8
9	AI246687	CTSC	Cathepsin C	286.7	529.8	1330.6	1541.0
10	NM_002835	PTPN12	Protein tyrosine phosphatase, non-receptor type 12	65.3	87.5	281.6	718.5

[a] Listed are the 10 most differentially expressed genes between CD8 T cell subsets. Mean expression values (data set 1: $n = 2$; data set 2: $n = 4$) are shown.

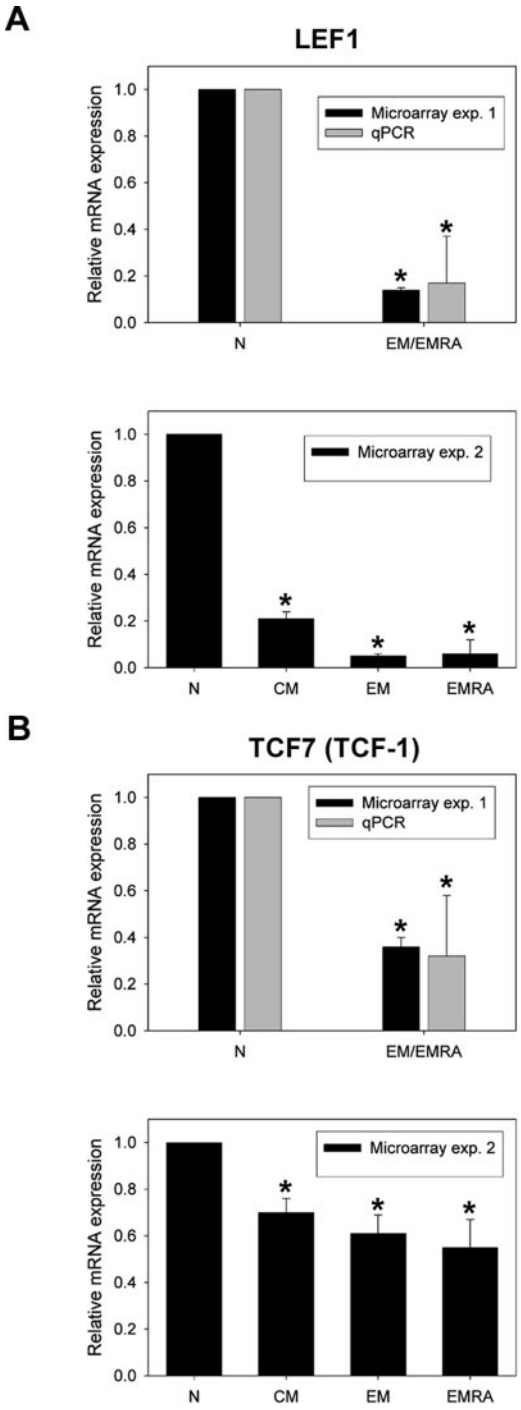

FIGURE 1. $CD8^+$ T_N down-regulate expression of LEF1 and TCF7 (TCF-1) mRNA upon Ag encounter in vivo. *Upper panels,* Expression of LEF1 (*A*) and TCF7 (TCF-1) (*B*) mRNA as examined in T_N ($CCR7^+CD45RA^+$) and T_{EM}/T_{EMRA} ($CCR7^-CD45RA^{+/-}$) CD8 T cell subsets by microarray analysis (data set 1, $n = 2$) and qRT-PCR ($n = 4$). *, $p < 0.05$ (two-sample t test with unequal variances), compared with T_N. In the second microarray data set (*lower panels*), LEF1 (*A*) and TCF7 (TCF-1) (*B*) mRNA expression was determined in T_N ($CCR7^+CD45RA^+$), T_{CM} ($CCR7^+CD45RA^-$), T_{EM} ($CCR7^-CD45RA^-$), and T_{EMRA} ($CCR7^-CD45RA^+$) subsets from four individual donors. LEF1 and TCF7 (TCF-1) mRNA expression relative to T_N is displayed. *, $p < 0.05$ (one-way ANOVA, Tukey's significant difference post hoc test), compared with T_N.

later time points poststimulation, although it varied between donors. A further set of experiments corroborated these results: in addition to TCR triggering, homeostatic signals such as IL-15 also

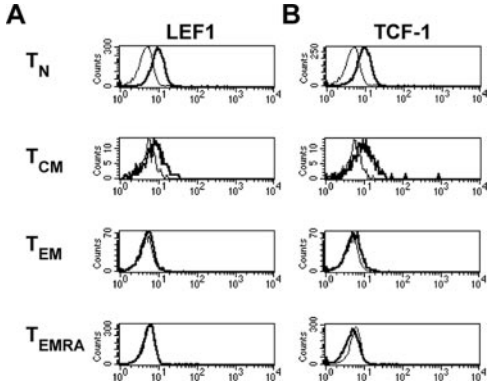

FIGURE 2. Intracellular LEF1 and TCF7 (TCF-1) protein expression in T_N and Ag-primed CD8 T cell subsets. LEF1 (*A*) and TCF7 (TCF-1) (*B*) protein expression was detected in CD8 T cell subsets by intracellular FACS staining as described in *Materials and Methods*. Thin line histograms indicate isotype control staining, thick line histograms LEF1 (*A*) or TCF7 (TCF-1) (*B*) staining. A representative example from one donor is shown. Similar results were obtained for CD8 T cell subsets from two other donors.

decreased LEF1 and TCF7 (TCF-1) mRNA expression in $CD8^+$ T_N (from CB) (Fig. 4*C*). The level of LEF1 down-regulation (5- to 10-fold) was similar to that observed when comparing T_N and CD8 T cells primed with Ag in vivo (Fig. 1*A*). Thus, the signals that control mature CD8 T cell differentiation, i.e., TCR triggering and homeostatic cytokines, also regulate the expression of LEF1 and TCF7 (TCF-1) in $CD8^+$ T_N.

TCR and IL-15 stimulation of peripheral CD8 T cells lead to a relative decrease in inhibitory LEF1 and TCF7 (TCF-1) isoform expression

Several LEF1 and TCF7 (TCF-1) isoforms with different functional properties have been described (13–21), but which of these different isoforms are expressed in CD8 T cells is unknown. Therefore, we analyzed LEF1 and TCF7 (TCF-1) isoform expression in mature T cells. First, cloning of LEF1 mRNA isoforms by RT-PCR and DNA sequencing showed that peripheral CD8 T cells express both stimulatory full-length and inhibitory ΔCTNNB LEF1 mRNA isoforms (Fig. 5*A*). Furthermore, we found that CD8 T cells preferentially express N-tail LEF1 mRNA isoforms, although isoforms carrying a B-tail could also be detected (Fig. 5*B*). These mRNA isoforms were distinguished by the presence (N-tail isoforms) or absence (B-tail isoforms) of exon 11 (16). The predominance of N-tail isoforms applied to both full-length and ΔCTNNB LEF1 mRNA isoforms (Fig. 5*B*).

Second, we investigated LEF1 and TCF7 (TCF-1) protein isoform expression by Western blot. Jurkat cells, a transformed immature T cell line with a high proliferative capacity, predominantly expressed stimulatory full-length LEF1 isoforms of 48–55 kDa (Fig. 6*A*) as previously reported (17). In contrast, primary resting CD8 T cells showed predominant expression of a 38-kDa LEF1 protein isoform (Fig. 6*A*). It has previously been demonstrated that this 38-kDa LEF1 band corresponds to the ΔCTNNB LEF1 isoform that has a dominant-negative function in the WNT signaling pathway (17, 18). The predominance of inhibitory LEF1 protein isoforms also applied to resting $CD8^+$ T_N (from CB; Fig. 6*B*, *lane 1*). In line with our mRNA expression results (Fig. 4), there was an overall down-regulation of LEF1 protein expression following TCR triggering or stimulation with IL-15 in vitro (Fig. 6*B*, *lanes 2* and *3*).

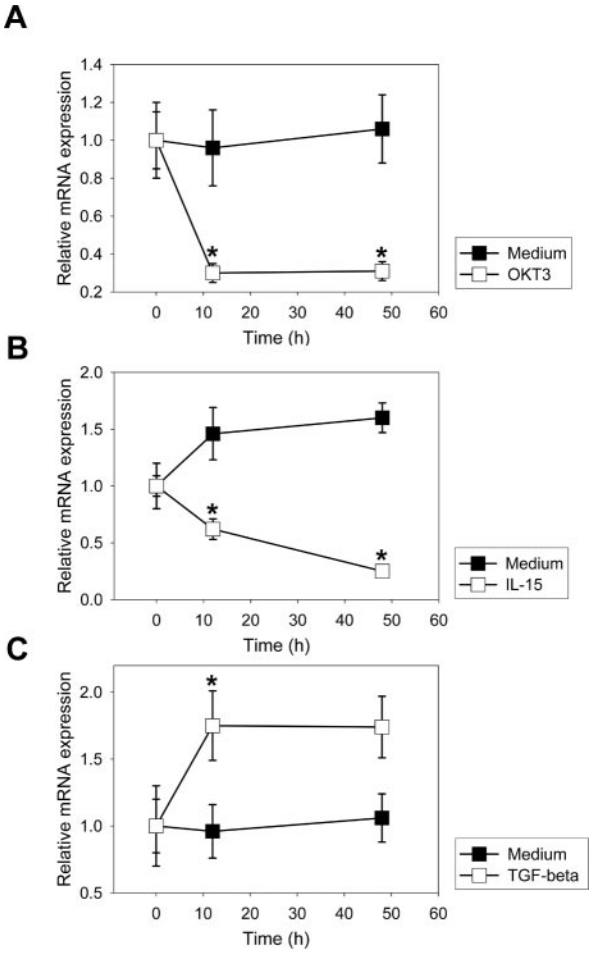

FIGURE 3. TCR signals and IL-15 down-regulate, while TGFβ1 up-regulates LEF1 mRNA expression in CD8 T cells. Human CD8 T cells were isolated by immunomagnetic selection and stimulated with either plate-bound anti-CD3 mAb/OKT3 at 1 μg/well (*A*), IL-15 at 50 ng/ml (*B*), or TGFβ1 at 3 ng/ml (*C*). At the indicated time points, LEF1 mRNA expression was examined by qRT-PCR relative to the resting state (= 0 h). *, $p < 0.05$ (two-sample *t* test with unequal variances), compared with medium control. Two independent experiments with CD8 T cells from different donors were performed.

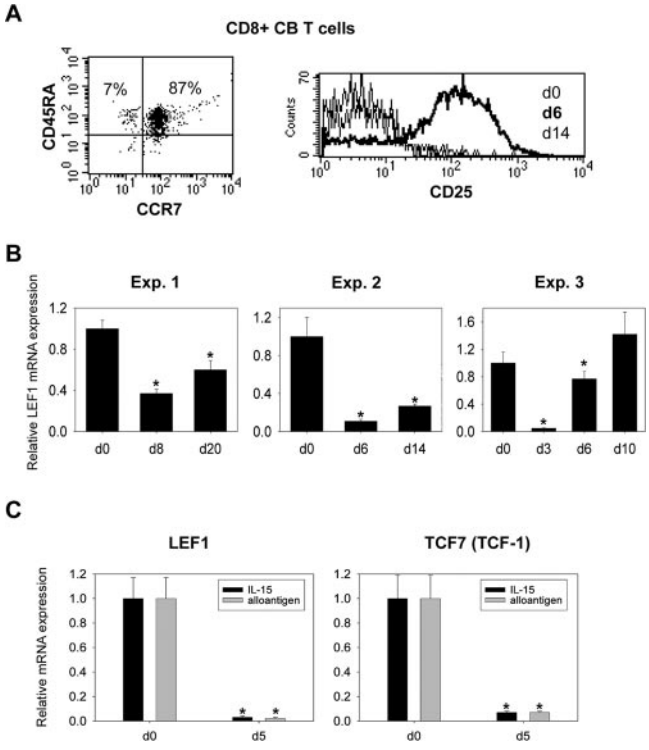

FIGURE 4. TCR and homeostatic signals inhibit expression of LEF1 and TCF7 (TCF-1) in CD8$^+$ T_N. *A,* Characterization of CB CD8 T cells. CD8 T cells were isolated from CB and their CCR7/CD45RA phenotype examined by FACS. A representative example from one donor is shown (*left panel*). The *right panel* shows expression of the activation marker CD25 (IL-2Rα) in resting CD8 T cells (day (d), d0), thin line histogram) and in cells poststimulation with alloantigen (d6, thick line histogram; d14, thin line histogram). *B,* LEF1 mRNA expression in response to allogeneic stimulation of CD8$^+$ T_N. CB CD8 T cells (*A*) were stimulated with irradiated allogeneic EBV-transformed B cells and LEF1 mRNA expression was determined by qRT-PCR at the indicated time points. Results from three independent experiments are shown. *, $p < 0.05$ (one-way ANOVA, Tukey's significant difference post hoc test), compared with day 0. *C,* Expression of LEF1 and TCF7 (TCF-1) mRNA in response to alloantigen and IL-15. CD8$^+$ T_N (from CB) were stimulated with either IL-15 at 50 ng/ml or with irradiated allogeneic EBV-transformed B cells for 5 days. Relative expression of LEF1 and TCF7 (TCF-1) as determined by qRT-PCR is shown for one of three independent experiments. *, $p < 0.05$ (two-sample *t* test with unequal variances), compared with day 0.

Similar to LEF1, we observed differences in the stimulatory vs inhibitory TCF7 (TCF-1) isoform balance between primary T cells and transformed Jurkat T cells. Jurkat cells showed predominant expression of stimulatory full-length TCF7 (TCF-1) isoforms of 42–48 kDa (Fig. 7*A*). In contrast, the ratio of stimulatory full-length (42–48 kDa)/inhibitory ΔCTNNB (26–32 kDa) TCF7 (TCF-1) isoforms (14, 15) was about equal in resting (Fig. 7*A*) and CD8$^+$ T_N (from CB; Fig. 7*B*, *lane 1*). Clevers and colleagues (15) have previously demonstrated that the 26- to 32-kDa bands correspond to ΔCTNNB TCF7 (TCF-1) isoforms that have a dominant-negative function (19, 20). Interestingly, TCR and IL-15 stimulation of CD8$^+$ T_N (from CB) lead to preferential down-regulation of the inhibitory TCF7 (TCF-1) ΔCTNNB isoforms (Fig. 7*B*, *lanes 2* and *3*). Thus, the TCF7 (TCF-1) protein isoform balance in CD8 T cells changed in favor of the stimulatory isoforms following activation. In conclusion, our data suggest that the negative effects of inhibitory LEF1 and TCF7 (TCF-1) isoforms prevail in resting CD8 T cells. In activated cells this negative effect seems to be relieved by down-regulation of overall LEF1 protein expression and by specific down-regulation of inhibitory TCF7 (TCF-1) isoform expression.

Discussion

Ag-experienced T cells are better able than T_N to respond to Ag. This reflects both an increase in their frequency and a change in their state of differentiation. The molecular mechanisms that underpin the changes in cellular state and hence the development of T cell memory remain poorly understood. The capacity of transcription factors to modulate many different aspects of T cell function makes them an attractive candidate for study in this respect. In two separate microarray studies, we observed that the WNT pathway effector, LEF1, was expressed at higher levels in T_N, compared with Ag-experienced CD8 T cells. Indeed, in both studies, it proved to be one of the most differentially expressed genes and the most differentially expressed transcription factor between these two populations of cells. A second effector of the same pathway, TCF7 (TCF-1), was also differentially expressed.

The differential expression of these molecules in the different CD8 T cell subsets proved to be consistent and robust; it was confirmed at the level of mRNA using qRT-PCR and at the level of protein using intracellular staining in many different donors.

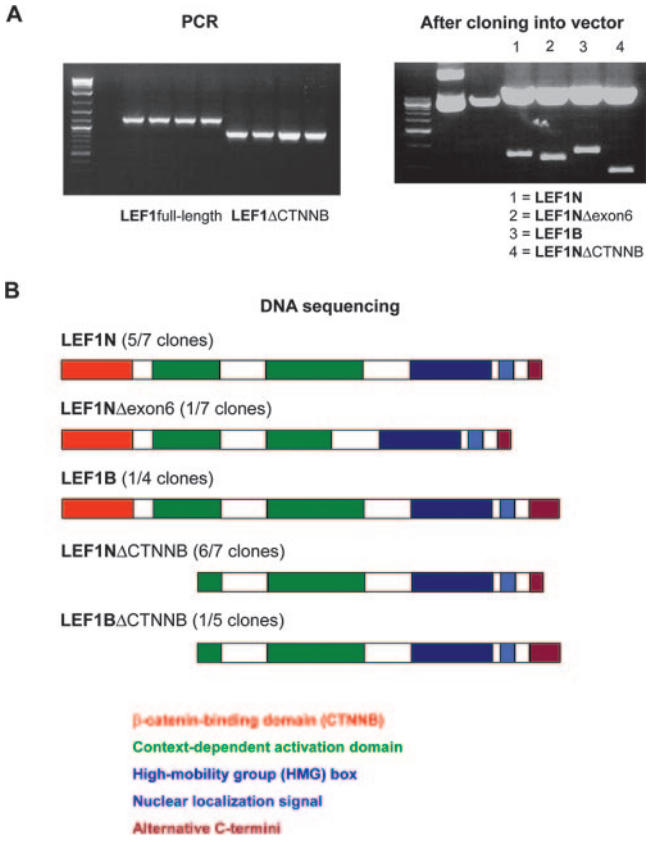

FIGURE 5. Expression of different LEF1 isoforms in primary CD8 T cells. *A,* Cloning of LEF1 isoforms from CD8 T cells. LEF1 mRNA isoforms were amplified from primary human CD8 T cells by RT-PCR using primers specific for either full-length or ΔCTNNB isoforms. *Left panel,* Gel electrophoresis of LEF1 PCR products. *First lane* on the *left,* DNA marker; and *second lane,* no template control. LEF1 PCR products were ligated into the vector pIRES2-EGFP and analyzed by restriction digestion (*right panel*). *First lane* on *left,* DNA marker; *second lane,* undigested parental vector (pIRES2-EGFP); and *third lane,* digested parental vector; *lanes 1–4,* digested pIRES2-LEF$_1$. *B,* CD8 T cells predominantly express N-tail LEF1 mRNA isoforms. DNA sequencing results for cloned LEF1 mRNA isoforms from *A* are displayed. The number of clones identical with a specific isoform among all of the clones sequenced is given in parentheses.

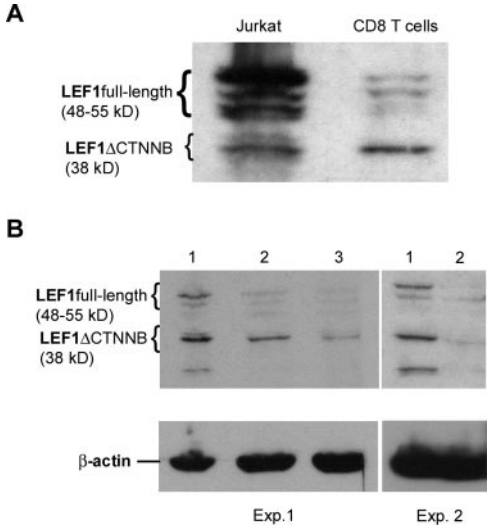

FIGURE 6. CD8$^+$ T$_N$ preferentially express inhibitory LEF1 isoforms and down-regulate LEF1 protein expression upon activation. *A,* Primary resting T cells show preferential expression of inhibitory LEF1 protein isoforms. Expression of full-length and truncated (ΔCTNNB) LEF1 protein isoforms in the Jurkat T cell line and in primary human CD8 T cells was examined by Western blotting. *B,* Down-regulation of LEF1 protein expression in activated CD8 T cells. CD8$^+$ T$_N$ (from CB) were either left unstimulated (*lane 1*) or stimulated with IL-15 at 50 ng/ml (*lane 2*) or alloantigen (*lane 3*). LEF1 protein expression was analyzed by Western blotting. Blotting with β-actin Ab (*bottom panel*) was used to assess protein loading. Two independent experiments are shown.

Furthermore, in a longitudinal study in an in vitro system, we were able to show that stimulation of T$_N$, by alloantigen, led to a down-regulation of LEF1 and TCF7 (TCF-1).

The importance of these two transcription factors in regulating thymocyte development is accepted. Their possible role in regulating peripheral T cell function has not been considered previously. T$_N$ can be regarded as peripheral stem cells, while T$_{EM}$ and T$_{EMRA}$ are differentiated cells with effector function. The idea that expression of LEF1 and TCF7 (TCF-1) may be relevant to maintaining the T$_N$ stem cell population is in line with the known role of the WNT-β-catenin-LEF1/TCF7 (TCF-1) pathway in the maintenance of hemopoietic stem cells (8–11). The observation that peripheral T cells from TCF7 (TCF-1)$^{-/-}$ mice have a spontaneously activated phenotype (CD44^{high}CD62L^{low}) that is characteristic of Ag-primed cells is also in line with this notion (23).

Our results are most consistent with the idea that LEF1 and TCF7 (TCF-1) might control T cell quiescence. Similar to TCR signals, we observed that other pro-proliferative signals such as the cytokines IL-2 and IL-15 inhibit LEF1 and TCF7 (TCF-1) expression. Interestingly, it has been shown that IL-15 and TCR stimulation induce very similar changes in gene expression in human CD8 T cells (30). This would suggest that IL-15 and TCR stimulation probably activate common signaling pathways, and this could also apply to the regulation of LEF1/TCF7 (TCF-1) expression in CD8 T cells. Furthermore, one recent study reported that another IL-2 family member, IL-7, can also inhibit LEF1 and TCF7 (TCF-1) expression (31). In contrast, we found that TGFβ1, which is known to inhibit T cell differentiation and maintain T cell quiescence (32), increased LEF1 expression. Interestingly, we noted a partial recovery of LEF1 expression in CD8 T cells in vitro when the cells converted back to a resting state following Ag stimulation. Similarly, one murine microarray study demonstrated initial down-regulation/partial recovery of LEF1 expression upon naive → effector → memory CD8 T cell differentiation in vivo (33).

The observed correlations between expression of LEF1 and TCF7 (TCF-1) and T cell naivety and quiescence were not consistent with published data showing that LEF1 and TCF7 (TCF-1) are able to drive cellular proliferation. However, this paradox was resolved by additional experiments that analyzed the expression of the different LEF1 and TCF7 (TCF-1) isoforms. Our work shows that, compared with Jurkat cells, resting CD8 T cells express relatively more of the inhibitory LEF1 and TCF7 (TCF-1) protein isoforms. T cell stimulation results in down-regulation of this inhibitory isoform. Importantly, it has previously been shown that although stimulatory full-length LEF1 and TCF7 (TCF-1) isoforms drive the proliferation of Jurkat T cells, dominant-negative ΔCTNNB isoforms inhibit proliferation (20). Furthermore, colon cancer cells predominantly express full-length LEF1 isoforms while down-regulating the expression of ΔCTNNB isoforms (17). Finally, inhibitory ΔCTNNB isoforms are the most abundant TCF7 (TCF-1) isoforms in the intestine and TCF7 (TCF-1)$^{-/-}$ mice develop intestinal and mammary adenomas (19). Thus, it is been suggested that the balance between stimulatory and inhibitory

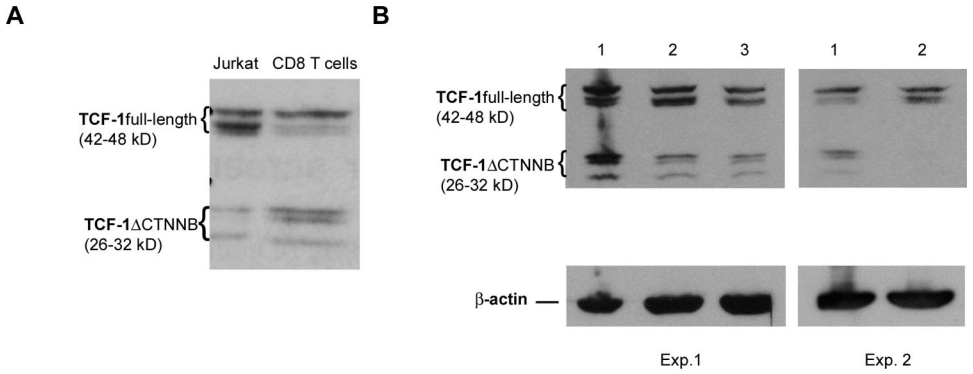

FIGURE 7. Activation of CD8$^+$ T_N changes the TCF7 (TCF-1) isoform balance in favor of stimulatory isoforms. *A,* Expression of TCF7 (TCF-1) protein isoforms in resting CD8 T cells. Expression of full-length and truncated (ΔCTNNB) TCF7 (TCF-1) protein isoforms in the Jurkat T cell line and in primary human CD8 T cells was examined by Western blotting. *B,* Preferential down-regulation of inhibitory TCF7 (TCF-1) isoform expression in activated CD8 T cells. CD8$^+$ T_N (from CB) were either left unstimulated (*lane 1*) or stimulated with IL-15 at 50 ng/ml (*lane 2*) or alloantigen (*lane 3*). TCF7 (TCF-1) isoform expression was analyzed by Western blotting. Two (of four) independent experiments are shown.

LEF1 and TCF7 (TCF-1) is a checkpoint for cellular proliferation in the context of malignancy.

In a conceptually similar way, the experiments we describe lead us to formulate the hypothesis that the balance between stimulatory and inhibitory LEF1 and TCF7 (TCF-1) isoforms represent a checkpoint for the quiescence of peripheral T cells. Direct evidence for this will require future studies, in which expression of individual LEF1 and TCF7 (TCF-1) isoforms or combinations of individual isoforms is manipulated in primary mature T cells. The functional redundancy between LEF1 and TCF7 (TCF-1) and the presence of numerous isoforms will make such experiments difficult to design and perform. Consistent with this, in preliminary experiments, we did not find a clear phenotype when knocking down total LEF1 (i.e., all isoforms) by RNA interference in human peripheral T cells. Studies in knockout and transgenic mice are probably more suited to address the role of the WNT pathway in mature T cells, although the conditional knockout of individual LEF1 and TCF7 (TCF-1) isoforms will be challenging.

In conclusion, our study identifies LEF1 as the most differentially expressed transcription factor between T_N and Ag-experienced CD8 T cells. It shows that, compared with a Jurkat cell line, CD8$^+$ T_N express more of the inhibitory isoform of this and another (TCF7 (TCF-1)) member of the WNT signaling pathway. We provide evidence that down-regulation of these inhibitory isoforms is associated with T cell stimulation. Our results suggest that the WNT pathway may have a specific function not only in immature, but also in mature T cells and provide a strong rationale for further molecular studies aimed at directly investigating the functional importance of individual isoforms of members of the WNT-LEF1/TCF7 (TCF-1) signaling pathway in peripheral T cell differentiation.

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Disclosures

The authors have no financial conflict of interest.

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